THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 5, Issue of January 31, pp. 2866–2875, 2003
Printed in U.S.A.

Dependence of Gonadotropin-releasing Hormone-induced Neuronal MAPK Signaling on Epidermal Growth Factor Receptor Transactivation*

Received for publication, August 27, 2002, and in revised form, November 13, 2002
Published, JBC Papers in Press, November 21, 2002, DOI 10.1074/jbc.M208783200

Bukhtiar H. Shah‡, Jae-Won Soh§, and Kevin J. Catt‡¶

From the ‡Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892 and §Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University, New York, New York 10032

The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), utilizes multiple signaling pathways to activate extracellularly regulated mitogen-activated protein kinases (ERK1/2) in normal and immortalized pituitary gonadotrophs and transfected cells expressing the GnRH receptor. In immortalized hypothalamic GnRH neurons (GT1–7 cells), which also express GnRH receptors, GnRH, epidermal growth factor (EGF), and phorbol 12-myristate 13-acetate (PMA) caused marked phosphorylation of ERK1/2. This action of GnRH and PMA, but not that of EGF, was primarily dependent on activation of protein kinase C (PKC), and the ERK1/2 responses to all three agents were abolished by the selective EGF receptor kinase inhibitor, AG1478. Consistent with this, both GnRH and EGF increased tyrosine phosphorylation of the EGF receptor. GnRH and PMA, but not EGF, caused rapid phosphorylation of the proline-rich tyrosine kinase, Pyk2, at Tyr^{402}. This was reduced by Ca^{2+} chelation and inhibition of PKC, but not by AG1478. GnRH stimulation caused translocation of PKCα and -ϵ to the cell membrane and enhanced the association of Src with PKCα and PKCϵ, Pyk2, and the EGF receptor. The Src inhibitor, PP2, the C-terminal Src kinase (Csk), and dominant–negative Pyk2 attenuated ERK1/2 activation by GnRH and PMA but not by EGF. These findings indicate that Src and Pyk2 act upstream of the EGF receptor to mediate its transactivation, which is essential for GnRH-induced ERK1/2 phosphorylation in hypothalamic GnRH neurons.

The hypothalamic decapeptide, gonadotropin releasing hormone (GnRH),[1] is a primary regulatory factor in the neuroendocrine control of reproduction and is released in an episodic manner from the hypothalamic GnRH neurons. The pulsatile delivery of GnRH to the anterior pituitary gland is essential to maintain the circulating gonadotropin profiles that are necessary for normal reproductive function. In addition to regulating pituitary gonadotropin release, GnRH has extrapituitary actions in neural and nonneural tissues and in several types of tumor cells (1). Immortalized GnRH-producing neurons (GT1–7 neurons) express several G protein-coupled receptors (GPCRs), including those for GnRH and luteinizing hormone/human chorionic gonadotropin (2, 3), as well as α- and β-adrenergic (4), muscarinic (5), and serotonergic receptors (6). These cells retain many of the characteristics of the native GnRH neurons, including the ability to maintain pulsatile GnRH release (1, 3). Recent evidence suggests that the autocrine action of GnRH on hypothalamic GnRH neurons is involved in the mechanism of pulsatile GnRH secretion (3).

Agonist activation of specific GPCRs and the resulting dissociation of their cognate G proteins releases α- and $\beta\gamma$-subunits that regulate phospholipase C-β, adenylyl cyclase, and ion channels, which in turn control the intracellular levels of inositol phosphates, Ca^{2+}, cAMP, and other second messengers (7, 8). The major signal transduction pathways in cells expressing GnRH receptors are initiated by activation of phospholipase C. The consequent calcium (Ca^{2+}) mobilization and activation of protein kinase C (PKC) by GnRH are key elements in the hypothalamic control of gonadotropin secretion from the anterior pituitary gland (1, 3, 5). Activation of PKC and Ca^{2+} mobilization during GnRH receptor stimulation are also responsible for mediating downstream signals leading to activation of extracellularly regulated mitogen-activated protein kinases (ERK1/2 MAPKs) that transmit signals from the cell surface to the nucleus to regulate transcriptional and other processes (7–13). However, the specific PKC isoforms that are involved in GnRH-induced ERK1/2 activation in GT1–7 cells are not known.

Mitogenic signaling by GPCRs can also occur through activation of tyrosine kinases of the Src family, focal adhesion kinases (FAKs), and receptor tyrosine kinases (RTKs). The RTKs involved in GPCR-mediated activation of ERK1/2 MAPKs include the EGF-R, platelet-derived growth factor receptor, and insulin-like growth factor receptor (14–16). The GPCRs mediating EGF-R transactivation during agonist stimulation include the AT_1 angiotensin receptor (17), the β-adrenoreceptor (18), the P2Y2 purinoceptor (19), and receptors for endothelin-1, thrombin, lysophosphatidic acid, and bradykinin (20, 21). GPCR-mediated transactivation of the EGF-R initiates the ERK1/2 MAPK cascade through recruitment of adaptor proteins, such as the Shc-Grb2-Sos complex, that activate the small G protein, Ras (14, 22). Depending on the GPCR

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: ERRB, NICHD, Bldg. 49, Rm. 6A36, National Institutes of Health, Bethesda, MD 20892-4510. Tel.: 301-496-2136; Fax: 301-480-8010; E-mail: catt@helix.nih.gov.

[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; Csk, C-terminal Src kinase; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; ERK1/2, extracellularly regulated MAPKs 1 and 2; ERK1/2-P, phosphorylated ERK1/2; GPCR, G protein-coupled receptor; PMA, phorbol 12-myristate 13-acetate; Pyk2, proline-rich tyrosine kinase; PKM, dominant-negative Pyk2 mutant; RTK, receptor tyrosine kinase; PKC, protein kinase C; FAK, focal adhesion kinase; PBS, phosphate-buffered saline; bFGF, basic fibroblast growth factor; HB-EGF, heparin-binding EGF; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

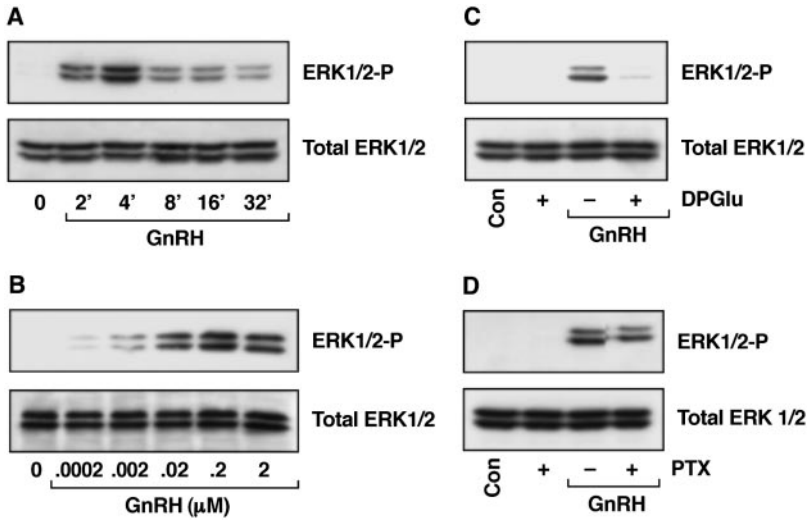

FIG. 1. **GnRH stimulation increases phosphorylation of ERK1/2 in a concentration-dependent manner.** *A*, time course of the effect of GnRH (200 nM) on ERK1/2 (p44 and p42) phosphorylation at Thr^{202}/Tyr^{204} (ERK1/2-P) in GT1–7 cells. Cells were grown to 70% confluence and serum-starved for 20–24 h. After GnRH treatment for selected times, cells were washed twice with ice-cold PBS and lysed in Laemmli sample buffer. Cells were sonicated, heated to 95 °C for 5 min, and centrifuged before loading onto 8–16% gradient gels for SDS-PAGE analysis. ERK1/2 phosphorylation (ERK1/2-P) was measured by immunoblotting with phospho-ERK1/2 antibodies. *B*, concentration-dependent effects of GnRH on ERK1/2 activation. Cells were treated with various concentrations of GnRH for 5 min. Blots were stripped and reprobed with ERK1/2 antibody as shown in the *lower panels*. *C*, the selective GnRH receptor antagonist [D-pGlu1,D-Phe,D-Trp(3,6)]GnRH (*DPGlu*) blocks the effect of GnRH on ERK1/2 activation. The antagonist (10 μM) was added 20 min before the addition of GnRH (200 nM) for 5 min. *D*, effect of pertussis toxin (*PTX*) on ERK1/2 activation by GnRH (200 nM for 5 min). Serum-starved cells were treated with PTX (50 ng/ml) for 24 h, and ERK1/2 activation was measured following stimulation with GnRH (200 nM for 5 min). *Con*, unstimulated control cells. All blots are representative of three or four experiments with similar results.

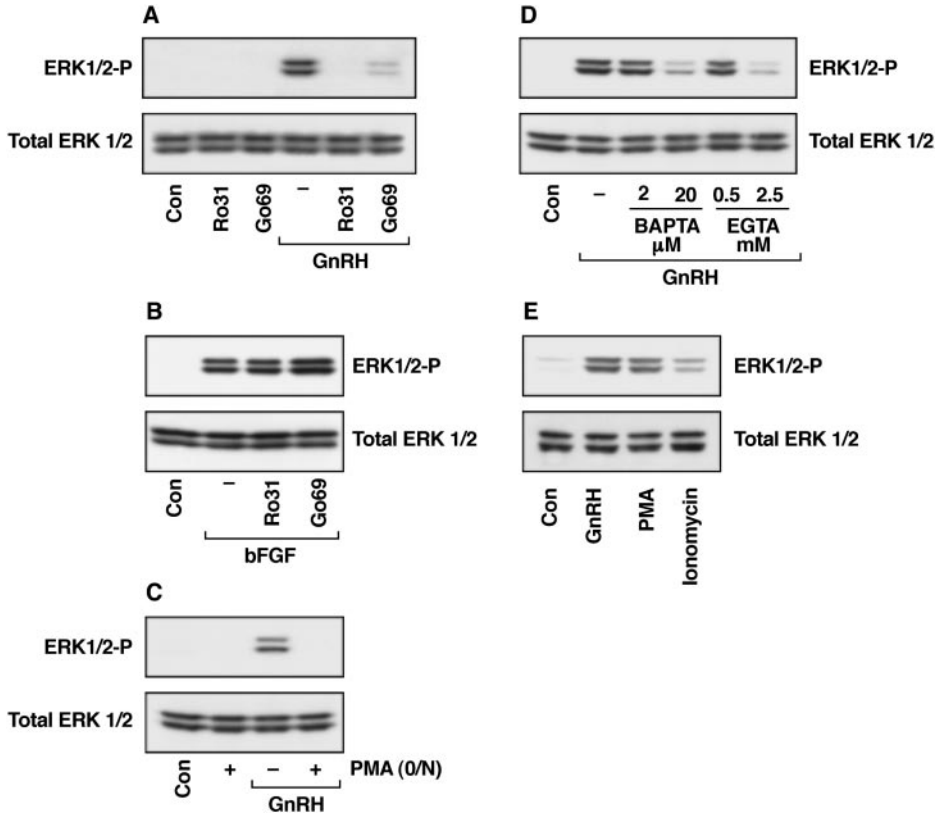

FIG. 2. **Role of PKC and calcium in GnRH-mediated ERK1/2 activation.** *A*, effects of PKC inhibitors on ERK1/2 activation by GnRH or bFGF in GT1–7 cells. *A* and *B*, cells were pretreated with Ro318220 (*Ro31*; 1 μM) and Go6983 (*Go69*; 1 μM) before stimulation with GnRH (200 nM for 5 min) or bFGF (10 ng/ml for 5 min). *C*, PKC depletion by overnight (*O/N*) PMA treatment (2 μM, 16 h) abolishes ERK1/2 activation by GnRH. *D*, effects of Ca^{2+} chelation on GnRH-induced ERK1/2 activation. Cells were treated with BAPTA (2 and 20 μM) or EGTA (0.5 and 2.5 mM) followed by stimulation with GnRH (200 nM) for 5 min. *E*, relative effects of GnRH (200 nM), ionomycin (10 μM), and PMA (100 nM) on ERK1/2 activation in GT1–7 cells. *Lower panels*, total ERK1/2 levels. *Con*, unstimulated control cells. All blots are representative of three experiments with similar results.

agonist and cell type, Ca^{2+}, PKC, G protein βγ subunits, and nonreceptor tyrosine kinases including Src and Pyk2, have been implicated in GPCR-induced EGF-R transactivation (14, 22). Endogenous EGF-Rs are expressed in several model systems, including αT3–1 gonadotrophs, COS-7 cells, and HEK-293 cells, that have been used in studies on GnRH signaling. However, the role of EGF-R transactivation in GnRH-induced ERK activation has been a subject of controversy and is not clearly defined (9, 10, 23). Also, the signaling molecules involved in cross-talk between the neuronal GnRH-R and the EGF-R have not been identified.

Depending upon the cell type, GPCRs mediate both Ras-independent ERK1/2 activation via stimulation of PKC and Ras-dependent ERK activation by receptor and nonreceptor tyrosine kinases (7, 14). GnRH has been found to activate ERK1/2 MAPKs in αT3–1 gonadotrophs and in COS-7 cells

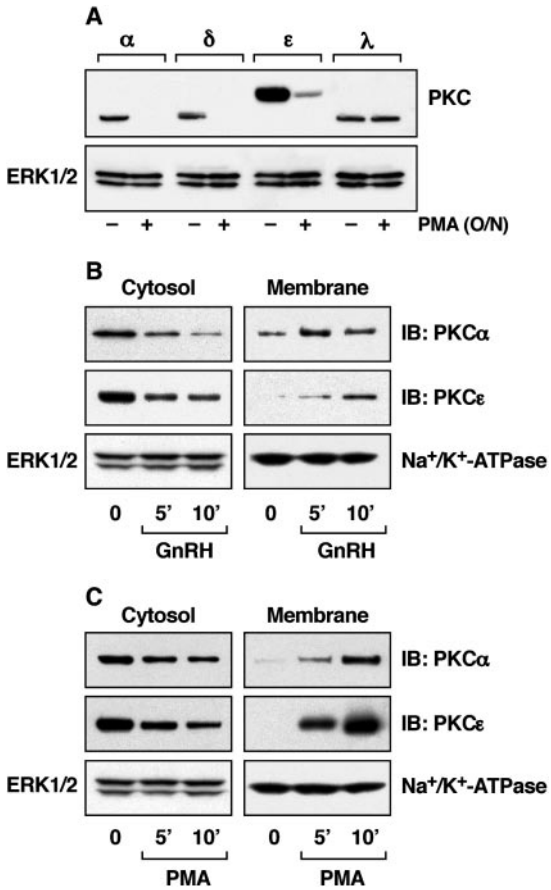

FIG. 3. **GnRH and PMA cause translocation of PKCα and -ϵ from cytosol to the membrane.** *A*, GT1–7 cells express PMA-sensitive and -insensitive PKC isoforms. Cells were treated with PMA (2 μM) overnight (*O/N*), and cell lysates were analyzed by Western blotting (*IB*) for various PKC isoforms. *Lower panel*, the ERK1/2 levels in control and PMA-treated cells. *B* and *C*, serum-starved cells were stimulated with GnRH (200 nM) and PMA (200 nM) for the time periods indicated. After washing with ice-cold PBS, cells were collected and homogenized. The cytosol and membranes were obtained as described under "Experimental Procedures." Equal amounts of proteins from control and stimulated cells were analyzed by SDS-PAGE and detected for PKC isoforms in cytosol and membranes. As controls, ERK1/2 and Na^+/K^+-ATPase were probed in cytosol and membranes, respectively. All data are representative of two or three experiments.

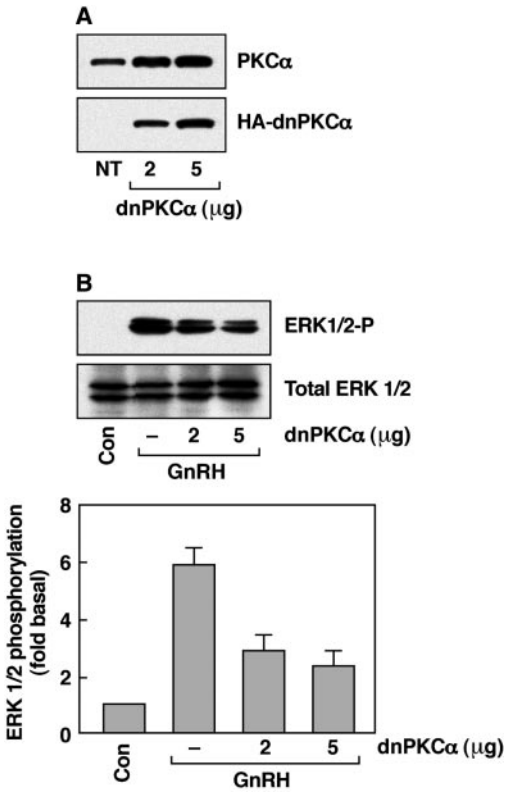

FIG. 4. **The effects of dominant negative PKCα on ERK1/2 activation by GnRH**. *A*, expression of native and transfected dominant negative PKCα (*dnPKCα*) in GT1–7 cells transfected with plasmid DNA (2 and 5 μg) encoding dominant negative PKCα. Cells were washed twice with ice-cold PBS and lysed in Laemmli sample buffer before loading onto 8–16% gradient gels for SDS-PAGE analysis. The expression of PKC constructs was detected using antibody against the hemagglutinin epitope with which these mutant proteins are tagged. Whereas conventional antibody against PKCα detects both native and exogenous dominant negative PKC proteins, the hemagglutinin antibody detects only the product of transfected dominant negative PKC with no immunoreactivity in the nontransfected (*NT*) cells. *B*, effects of overexpression of dominant negative PKCα on GnRH-induced ERK1/2 phosphorylation (*ERK1/2-P*). Serum-starved cells were stimulated with GnRH (200 nM for 5 min) and then washed twice with ice-cold PBS and lysed in Laemmli sample buffer before loading onto 8–16% gradient gels for SDS-PAGE analysis. The quantitated data are shown in the *lower panel* ($n = 4$).

(8–10, 12–13) and GH3 cells transfected with the GnRH receptor (11). It also stimulates Jun N-terminal kinase in αT3–1 cells (24) and p38-MAPK in LβT2 gonadotrophs (25). Activation of these MAPKs by other GPCRs, such as angiotensin II (26, 27), endothelin (28), adrenomedullin (29), and acetylcholine (30), is mediated through the proline-rich protein tyrosine kinase, Pyk2. In general, Pyk2 activation in conjunction with Src kinase appears to be a key element in GPCR-mediated transactivation of the EGF-R (31). However, no information is available on the role of Pyk2 and the nature of its interaction with Src and EGF-R during receptor stimulation by GnRH. The present studies have identified a signaling cascade that mediates GnRH-induced ERK1/2 phosphorylation in immortalized GnRH neurons (GT1–7 cells) and is dependent on receptor-mediated activation of PKC, Src, Pyk2, and the EGF-R.

EXPERIMENTAL PROCEDURES

Materials—GnRH was obtained from Peninsula Laboratories, Inc. (Belmont, CA), EGF was from Invitrogen, and pertussis toxin was from List Biological Laboratories. Protein assay kits were from Pierce. ERK1/2 and anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) antibodies were from New England Biolabs, and secondary antibodies conjugated to horseradish peroxidase were from KPL. Antibodies against Src, EGF-R, phospho-EGF-R (Tyr^{1173}), and phosphotyrosine (PY20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-Pyk2 (Tyr^{402}) was from either Calbiochem or BIOSOURCE International, and anti-phospho-EGF-R (Tyr^{1068}) was from BIOSOURCE International. Mouse monoclonal hemagglutinin tag antibody was from Covance Babco (Berkeley, CA). AG1478, Go6983, Ro318220, PP2, BAPTA, PMA, and wortmannin were from Calbiochem, and antibodies against PKC isoforms and Pyk2 were from Transduction Laboratories. LipofectAMINE was from Invitrogen. The Pyk2, dominant negative Pyk2, constitutively active Src, and Csk constructs were provided by Dr. Zvi Naor (University of Tel Aviv). PKC isoform-specific dominant negative and constitutively active constructs tagged with the hemagglutinin epitope were prepared as previously described (32). Western blotting reagents and ECL were obtained from Amersham Biosciences or Pierce.

Cell Culture and Transfections—GT1–7 neurons donated by Dr. Richard Weiner (University of California, San Francisco) were grown in culture medium consisting of 500 ml of Dulbecco's modified Eagle's medium containing 0.584 g/liter L-glutamate and 4.5 g/liter glucose, mixed with 500 ml of F-12 medium containing 0.146 g/liter L-glutamate, 1.8 g/liter glucose, 100 μg/ml gentamicin, 2.5 g/liter sodium carbonate, and 10% heat-inactivated fetal calf serum. DNA transfections were performed with LipofectAMINE according to the manufacturer's instructions.

Inositol Phosphate Measurements—Cells were labeled for 24 h in inositol-free Dulbecco's modified Eagle's medium containing 20 μCi/ml [^{3}H]inositol as previously described (5) and then washed twice with

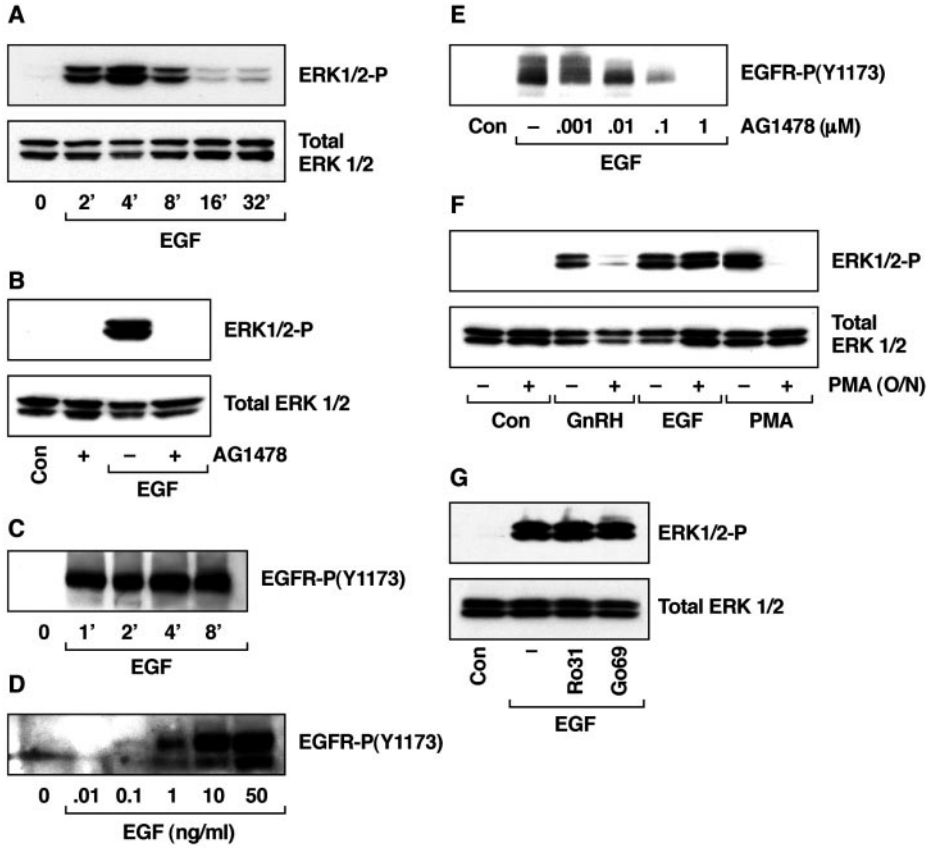

FIG. 5. **EGF causes marked phosphorylation of ERK1/2 and EGF-R in GT1–7 cells.** *A*, cells were treated with EGF (50 ng/ml) for the time periods indicated, and ERK1/2 phosphorylation was determined as described under "Experimental Procedures." *B*, the EGF receptor kinase inhibitor, AG1478 (100 nM) completely inhibits ERK1/2 activation by EGF (50 ng/ml). *C*, time course effect of EGF (50 ng/ml) on EGF-R phosphorylation(EGF-R-P)atTyr^{1173}.*D*,concentration-dependent effects of EGF (4-min stimulation) on EGF-R phosphorylation at Tyr^{1173}. Serum-starved GT1–7 cells treated with EGF were collected in Laemmli sample buffer and analyzed for immunoblotting with anti-phosphotyrosine EGF-R antibody at Tyr^{1173}. *E*, concentration-dependent inhibitory effect of AG1478 on EGF-induced phosphorylation of the EGF-R at Tyr^{1173}. *F*, PKC depletion by PMA treatment (2 μM) overnight (*O/N*) abolishes ERK1/2 activation induced by GnRH (200 nM for 5 min) and PMA (100 nM for 8 min) but not by EGF (50 ng/ml). *G*, lack of effect of PKC inhibitors, Ro318220 (*Ro31*; 1 μM) and Go6983 (*Go69*; 1 μM) on EGF-induced ERK1/2 activation. GT1–7 cells were pretreated with inhibitors for 20 min and stimulated with EGF (50 ng/ml) for 5 min.

inositol-free M199 medium and stimulated at 37 °C in the presence of 10 mM LiCl. The reactions were stopped with perchloric acid, inositol phosphates were extracted, and radioactivity was measured by liquid scintillation γ-spectrometry.

Subcellular Fractionation—Serum-starved GT1–7 cells were treated with either PMA or GnRH for the times indicated and then washed twice with ice-cold PBS and collected in homogenization buffer containing 25 mM Tris·HCl, pH 7.4, 2 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin and leupeptin. After they were kept on ice for 10 min, cells were homogenized with 25 strokes of a Dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation at 500 g for 5 min, and the supernatant was centrifuged at 100,000 × *g* for 30 min. The high speed supernatant constituted the cytosolic fraction. The pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 30 min. The Triton-soluble component (membrane fraction) was separated from the insoluble material (cytoskeletal fraction) by centrifugation at 100,000 × *g* for 20 min.

Immunoprecipitation—After treatment with inhibitors and drugs, cells were placed on ice, washed twice with ice-cold PBS, lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM NaF, 0.25% sodium deoxycholate, 1 mM EDTA, 1% Nonidet P-40, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstain, and 1 mM 4-(2-aminoethyl)benzensulfonyl fluoride), and probe-sonicated (Sonifier cell disruptor). Solubilized lysates were clarified by centrifugation at 8000 × *g* for 10 min, precleared with agarose, and then incubated with specific antibodies and protein A- or G-agarose. The immunoprecipitates were collected, washed four times with lysis buffer, and dissolved in Laemmli buffer. After heating at 95 °C for 5 min, the samples were centrifuged briefly, and the supernatants were analyzed by SDS-PAGE on 8–16% gradient gels.

Immunoblot Analysis—Cells grown in six-well plates and at 60–70% confluence were serum-starved for 24 h before treatment at 37 °C with selected agents. The media were then aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 μl of Laemmli sample buffer. The samples were briefly sonicated, heated at 95 °C for 5 min, and centrifuged for 5 min. The supernatant was electrophoresed on SDS-PAGE (8–16%) gradient gels and transferred to polyvinylidene difluoride membranes. Blots were incubated overnight at 4 °C with primary antibodies and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with enhanced chemiluminescence reagent (Amersham Biosciences or Pierce) and quantitated with a laser-scanning densitometer. In some cases, blots were stripped and reprobed with other antibodies.

RESULTS

GnRH treatment of GT1–7 cells caused transient stimulation of ERK1/2 that reached a peak at 5 min and declined thereafter toward the basal level over 30 min (Fig. 1*A*). GnRH-induced ERK1/2 activation was concentration-dependent over the 0.2–200 nM range and was abolished by the GnRH receptor antagonist, [D-pGlu1,D-Phe,D-Trp(3,6)]GnRH (Fig. 1, *B* and *C*). GnRH receptors are primarily coupled to $G_{q/11}$ proteins, but some of the physiological actions of GnRH are known to occur through activation of G_s or G_i proteins (5, 33). In GT1–7 neurons, nanomolar GnRH concentrations cause marked elevation of inositol phosphate production through G_q-mediated activation of phospholipase C and also stimulate cAMP production. Higher concentrations of GnRH (0.1–1 μM) reduce intracellular cAMP levels in a pertussis toxin-sensitive manner, suggesting that GnRH activates a G_i protein in GT1–7 cells (34). Consistent with this, pertussis toxin had a modest inhibitory effect (~30%) on GnRH-induced ERK activation, suggesting partial involvement of G_i protein(s) in MAPK signaling in GT1–7 cells (Fig. 1*D*).

The roles of PKC and Ca^{2+} in agonist-stimulated activation of ERK1/2 were evaluated in studies with PKC inhibitors and the Ca^{2+} chelators BAPTA-2AM and EGTA. GnRH-induced ERK1/2 activation was found to be highly PKC-dependent and was abolished by the PKC inhibitors Ro318220 and Go6983 (Fig. 2*A*). These inhibitors had no effect on ERK1/2 activation induced by basic fibroblast growth factor (bFGF) (Fig. 2*B*) or isoproterenol, a β_2-adrenoreceptor agonist (data not shown). Consistent with its critical role in GnRH action, depletion of PKC by prolonged PMA treatment (1 μM for 16 h) abolished agonist-induced ERK1/2 activation (Fig. 2*C*). However, ERK1/2

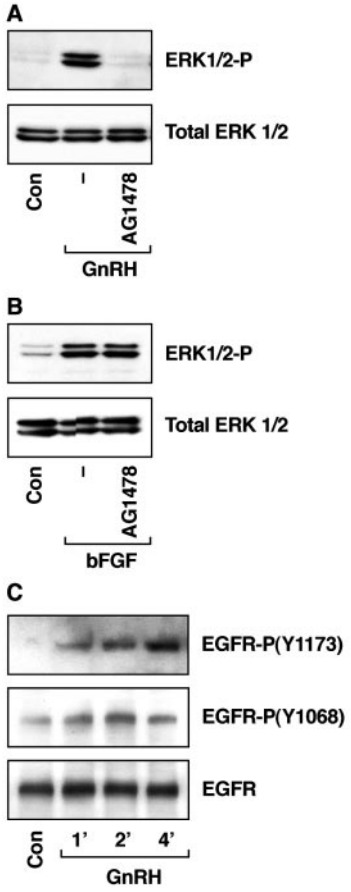

FIG. 6. **GnRH-induced ERK1/2 activation is dependent on EGF-R transactivation in GT1–7 cells.** *A*, the EGF receptor kinase inhibitor, AG1478 (100 nM), inhibits ERK1/2 activation by GnRH (100 nM, 5 min). *B*, lack of inhibitory effect of AG1478 on ERK1/2 activation induced by bFGF (10 ng/ml for 5 min). *C*, time course effects of GnRH on phosphorylation of the EGF-receptor. Serum-starved cells were stimulated with GnRH (200 nM), collected in Laemmli sample buffer, and analyzed for immunoblotting with anti-phosphotyrosine EGF-R antibody at Tyr^{1173} or Tyr^{1068}. The *lower panel* shows total EGF-R protein.

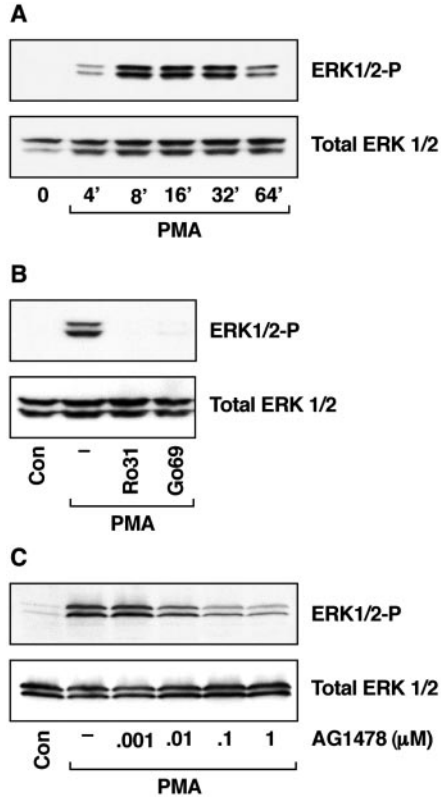

FIG. 7. **The involvement of EGF-R transactivation in PMA-induced ERK1/2 activation.** *A*, time course effect of PMA (100 nM) on ERK1/2 activation. *B*, effects of PKC inhibitors, Ro318220 (*Ro31*; 1 μM) and Go6983 (*Go69*; 1 μM) on PMA-induced ERK1/2 activation. *C*, effect of dominant negative PKCα mutant (2 μg of DNA) on PMA-induced ERK1/2 activation. Serum-starved GT1–7 cells were pretreated with inhibitors for 15 min before stimulation with PMA (100 nM) for 8 min. The blots were reprobed with nonphosphorylated ERK1/2, and data are shown in the *lower panels* (n = 3–4).

activation by GnRH was less sensitive to Ca^{2+} chelation by EGTA and BAPTA (Fig. 2*D*). Consistent with this, the PKC activator, PMA, was much more effective than the Ca^{2+} ionophore, ionomycin, in eliciting ERK1/2 activation (Fig. 2*E*). These findings suggest that GnRH receptor-mediated ERK1/2 activation in GT1–7 cells is predominantly dependent on PKC.

A major role of PKC in GnRH-induced ERK activation has also been reported in other cell types (8–10), but little information is available about the involvement of specific PKC isoforms in this cascade. GT1–7 cells were found to contain several immunoreactive PKC isoforms, including α, δ, ε, and λ. Overnight PMA stimulation (2 μM) caused down-regulation of PKCα, -δ, and -ε but not PKCλ (Fig. 3*A*). Among the PMA-sensitive PKC isoforms, only PKCα and -ε were translocated from cytosol to the cell membrane during treatment with GnRH and PMA (Fig. 3*B*). These effects of GnRH and PMA were specific, since no changes in the levels of ERK1/2 and Na^+/K^+-ATPase were found in cytosol and membranes, respectively (Fig. 3*C*). The predominant role of PKCα in GnRH-induced ERK activation was confirmed in studies with constitutively active and dominant negative mutants of PKCα. These results showed that GnRH-induced ERK1/2 phosphorylation was attenuated by dominant negative PKCα (*dnPKCα*; Fig. 4) and was increased with transfection of constitutively active PKCα (data not shown).

It is well established that transactivation of receptor tyrosine kinases such as the EGF-R contributes to GPCR-mediated ERK1/2 activation in certain cell types (14, 22). However, studies on the role of the EGF-R in GnRH action have not given consistent results (9, 10). Thus, whereas Grosse *et al.* (10) implicated transactivation of the EGF-R in GnRH-induced stimulation of ERK1/2 phosphorylation in αT3–1 pituitary gonadotrophs, Benard *et al.* (9) subsequently reported that the major pathway of ERK1/2 activation was through PKC and activation of Raf-1 and did not involve the EGF-R. Since GT1–7 cells express receptors for both EGF and GnRH, we evaluated the role of the EGF-R in GnRH-induced MAPK signaling. In this cell type, EGF, like GnRH, caused transient activation of ERK1/2 (Fig. 5*A*). As expected, the selective EGF-R kinase inhibitor, AG1478, blocked the ERK1/2 activation induced by EGF (Fig. 5*B*). EGF stimulation caused rapid and marked phosphorylation of the EGF-R at Tyr^{1173} in a time- and concentration-dependent manner (Fig. 5, *C* and *D*). Our data suggest a potential role of PKC in GnRH-induced ERK1/2 activation. To determine whether PKC acts upstream or downstream of the EGF-R, we examined the effect of PKC inhibition on EGF-induced ERK1/2 activation. Whereas PKC depletion by prolonged PMA treatment or PKC inhibitors abolished the effects of PMA and GnRH, it had no effect on EGF responses (Fig. 5, *E* and *F*). These data indicate that EGF-induced ERK1/2 activation is PKC-independent and that PKC acts upstream of the EGF-R during GnRH signaling in GT1–7 cells.

To examine the involvement of EGF-R in GnRH-induced ERK1/2 activation in GT1–7 cells, cells were pretreated with AG1478 and stimulated with GnRH (200 nM for 5 min). As

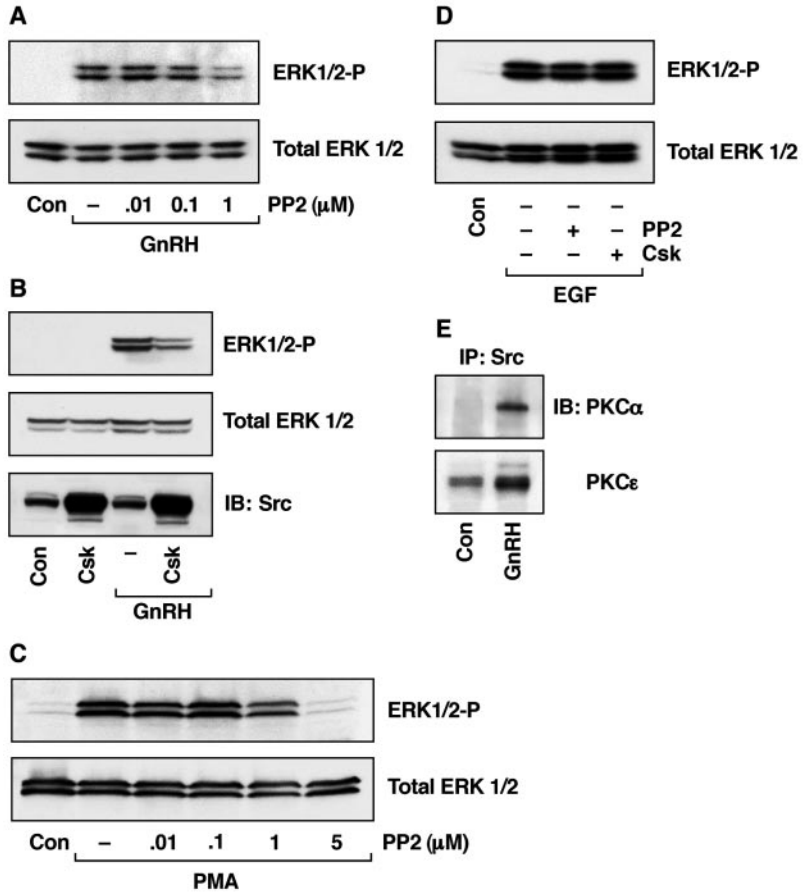

FIG. 8. **Role of Src in GnRH-induced ERK1/2 activation.** *A*, concentration-dependent effect of the Src kinase inhibitor, PP2, on ERK1/2 activation induced by GnRH. Serum-starved GT1–7 cells were incubated with PP2 (0.01–1 μM) for 20 min and then stimulated with GnRH (200 nM) for 5 min. *B*, effect of Src negative regulatory kinase (Csk) on ERK1/2 activation induced by GnRH. Csk DNA (1 μg) was transfected in GT1–7 cells, and ERK1/2 activation was measured following stimulation with GnRH (100 nM for 5 min). Blots were reprobed with Src antibody. *C*, concentration-dependent effects of Src kinase inhibitor, PP2, on PMA-induced ERK1/2 activation. Serum-starved cells were treated with PP2 for 20 min before stimulation with PMA (100 nM for 8 min). *D*, lack of effects of PP2 (5 μM) and Csk overexpression on EGF-induced ERK1/2 phosphorylation. *E*, GnRH stimulation increases association of PKCα and -ϵ with Src. Serum-starved cells were stimulated with GnRH (200 nM) for 5 min, washed with ice-cold PBS, collected in lysis buffer, immunoprecipitated (*IP*) with Src antibody, and immunobloted (*IB*) with antibodies against PKCα and -ϵ (n = 3).

shown in Fig. 6*A*, GnRH-stimulated ERK1/2 phosphorylation was also abolished by AG1478, indicating its absolute dependence on transactivation of the EGF-R. The inhibitory action of AG1478 on GnRH-induced ERK1/2 activation was selective and did not affect bFGF-stimulated ERK1/2 phosphorylation (Fig. 6*B*). Consistent with the role of EGF-R in GnRH signaling, GnRH also stimulated phosphorylation of the EGF-R as measured with anti-phosphopeptide antibodies that recognize the phosphorylated molecule at Tyr^{1173} or Tyr^{1168} (Fig. 6*C*), the major sites of Src kinase phosphorylation (35) and Grb2 binding (36), respectively. These data demonstrate that transactivation and phosphorylation of the EGF-R are essential for GnRH signaling through ERK1/2 in GT1–7 cells.

Since GnRH-induced ERK1/2 activation is primarily dependent on PKC and GnRH causes PKC activation through generation of diacylglycerol (8–10), we investigated the effects of PMA on this cascade. The results revealed that PMA caused marked ERK1/2 activation that was abolished by prior PKC depletion (as shown above in Fig. 5*D*) and by PKC inhibitors, Ro318220 and Go6983 (Fig. 7, *A* and *B*). To determine whether PMA mimics the effects of GnRH with respect to EGF-R transactivation, GT1–7 cells were treated with AG1478 and stimulated with PMA. As shown in Fig. 7*C*, PMA-induced ERK1/2 activation was extinguished in a dose-dependent manner by the EGF-R kinase inhibitor, AG1478, indicating that GnRH-induced ERK1/2 activation occurs through EGF-R transactivation in a PKC-dependent manner.

Since there is no consensus on the types of intermediate proteins involved during GPCR-induced transactivation of the EGF-R (14, 36, 37), we examined the roles of Src and Pyk2 in GnRH-induced EGF-R phosphorylation and ERK activation. In GT1–7 cells, the highly selective Src kinase inhibitor, PP2, and the Src negative regulatory kinase, Csk, attenuated the activation of ERK1/2 by GnRH (Fig. 8, *A* and *B*). Similarly, Src inhibition abolished the effect of PMA on ERK1/2 activation (Fig. 8*C*). In contrast, Src inhibition and Csk had no effect on EGF-induced ERK1/2 responses (Fig. 8*D*). These data suggest that Src has a critical role in GnRH-induced activation of the EGF-R and ERK1/2. Since our data show that both PKC and Src act upstream of EGF-R, we examined the interaction between PKC and Src. As shown in Fig. 8*E*, GnRH stimulation increased the association of PKCα and -ϵ with Src.

A role for the nonreceptor proline-rich tyrosine kinase, Pyk2, in ERK1/2 activation by some GPCRs has been shown (26–30). However, nothing is known about the role of Pyk2 in GnRH signaling. GnRH stimulation of GT1–7 cells caused a marked increase in Pyk2 tyrosine phosphorylation at residue 402 that commenced within 1 min and was sustained for up to 30 min (Fig. 9*A*). Like ERK1/2 activation, the stimulatory effect of GnRH on Pyk2 activation was sensitive to both PKC inhibition and Ca^{2+} chelation (Fig. 9*B*). Consistent with the potential involvement of PKC in this cascade, PMA also caused phosphorylation of Pyk2 at Tyr^{402}, and this effect was attenuated by PKC inhibition but not by AG1478 (Fig. 9*C*). These data suggest that GnRH-induced Pyk2 activation is primarily PKC-dependent in GT1–7 cells.

The dependence of GnRH-mediated ERK1/2 activation on Pyk2 was evaluated in studies with dominant negative Pyk2 mutants (PKMs). Overexpression of PKM attenuated the stimulatory effects of GnRH and PMA on ERK1/2 activation, and Pyk2 overexpression enhanced the effect of GnRH on ERK1/2 activation (Fig. 10*A*). These data show that Pyk2 has an important role in GnRH-induced ERK1/2 activation in GT1–7 cells. Previous studies have shown that, depending on the cell

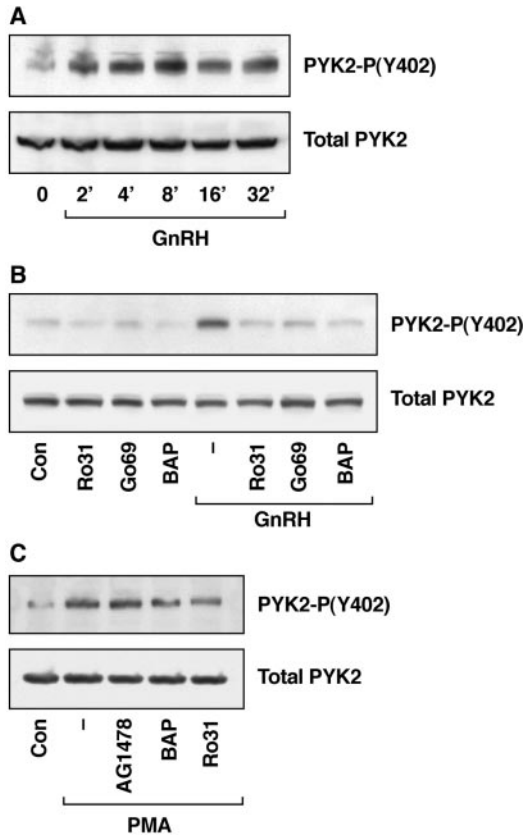

FIG. 9. **GnRH and PMA cause Pyk2 phosphorylation in GT1–7 cells.** *A*, serum-starved GT1–7 cells were stimulated with GnRH (200 nM) for the time periods indicated, and cell lysates were analyzed for Pyk2 activation (Pyk2-P) using phosphotyrosine antibody to Pyk2 (Tyr^{402}). *B*, effects of PKC inhibitors and Ca^{2+} chelation on Pyk2 activation by GnRH. Serum-starved GT1–7 cells were treated with Ro318220 (*Ro31*; 1 μM), Go6983 (*Go69*; 1 μM), and BAPTA (*BAP*; 10 μM) for 20 min before stimulation with GnRH (200 nM, 5 min). *C*, effects of AG1478 (100 nM), calcium chelator, BAPTA (*BAP*; 10 μM), and PKC inhibitor, Ro318220 (*Ro31*; 1 μM) on PMA-induced phosphorylation of Pyk2 (*Pyk2-P*). GT1–7 cells were incubated with inhibitors for 20 min and stimulated with PMA (100 nM) for 8 min. The blot was reprobed with ERK1/2 antibody to show the equal loadings of the protein in each lane. Data are representative of three independent experiments.

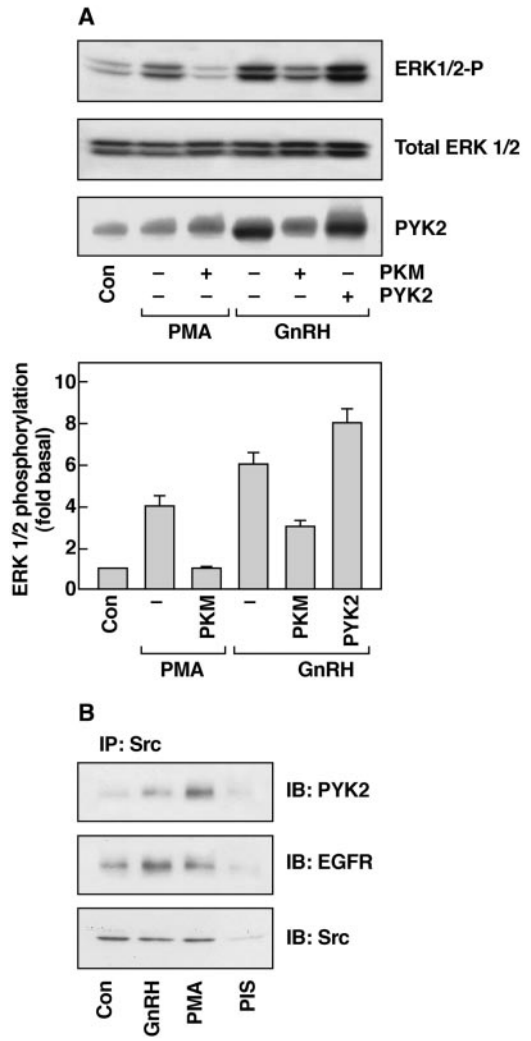

FIG. 10. **Role of Pyk2 in GnRH-induced ERK activation.** *A*, effects of overexpression of Pyk2 dominant negative mutant, PKM, and Pyk2 (1 μg DNA) on ERK1/2 activation induced by PMA (100 nM for 8 min) and GnRH (200 nM for 5 min). Blots were stripped and reprobed with ERK1/2 and Pyk2 antibodies. The data from three experiments were quantitated. *B*, effects of GnRH (200 nM) and PMA (100 nM) on the association of Src with Pyk2 and EGF-R. Agonist-stimulated cells were collected in lysis buffer, immunoprecipitated (*IP*) with Src antibody or preimmune serum (*PIS*), and immunoblotted (*IB*) with either Pyk2 or EGF-R antibody. Data are representative of two experiments.

types, GPCR stimulation leads to interaction of Src with Pyk2 and also that these proteins can cause activation of one another (20, 37). Whether such an interaction occurs following GnRH stimulation is not known. An analysis of the cell lysates immunoprecipitated with anti-Src antibody and immunoblotted with Pyk2 antibody revealed that GnRH increased the association of Src with Pyk2. Furthermore, Src also co-immunoprecipitated with the EGF-R in GT1–7 cells (Fig. 10*B*). Taken together, these results indicate that ERK1/2 activation by GnRH leads to recruitment of a multicomponent signaling complex that includes PKCα/ϵ, Src, Pyk2, and the EGF-R.

In contrast, although EGF caused marked activation of ERK1/2, it had no effect on Pyk2 phosphorylation (Fig. 11*A*). Moreover, Pyk2 activation by GnRH was not prevented by AG1478 (Fig. 11*B*), indicating that GnRH-induced Pyk2 activation precedes that of the EGF-R transactivation. Whereas PKM decreased ERK1/2 activation by GnRH and PMA, it had no effect on EGF-induced ERK1/2 activation and EGF-R phosphorylation (Fig. 11, *C* and *D*). Because both Pyk2 and FAK show high sequence similarity, cellular localization, and signaling characteristics (38, 39), we determined whether GnRH-induced ERK1/2 activation also involves FAK activation. In contrast to its marked effect on Pyk2 activation, GnRH had little effect on FAK phosphorylation (data not shown). These data suggest that GnRH causes selective activation of Pyk2 through its specific receptors in GT1–7 cells.

DISCUSSION

The activity of the mammalian pituitary-gonadal axis is dependent on the pulsatile secretion of GnRH from hypothalamic GnRH neurons. Many of the genomic effects of GnRH in its neuroendocrine target cells are believed to be mediated by the activation of MAPKs, which convey GnRH signaling from the cell surface to the nucleus for regulation of genes controlling the functions of GnRH neurons and pituitary gonadotropes (1, 8). However, the roles of intermediate signaling molecules such as Pyk2 and the EGF-R in this pathway have not been clearly defined. Our results show that GnRH causes rapid, marked, and transient phosphorylation of ERK1/2 through transactivation of the EGF-R in GT1–7 cells. The signaling response to GnRH also involves the PKC-dependent phosphorylation of two nonreceptor tyrosine kinases, Src and Pyk2. To date, there has been no indication that this mechanism, with consequent transactivation of the EGF-R, is

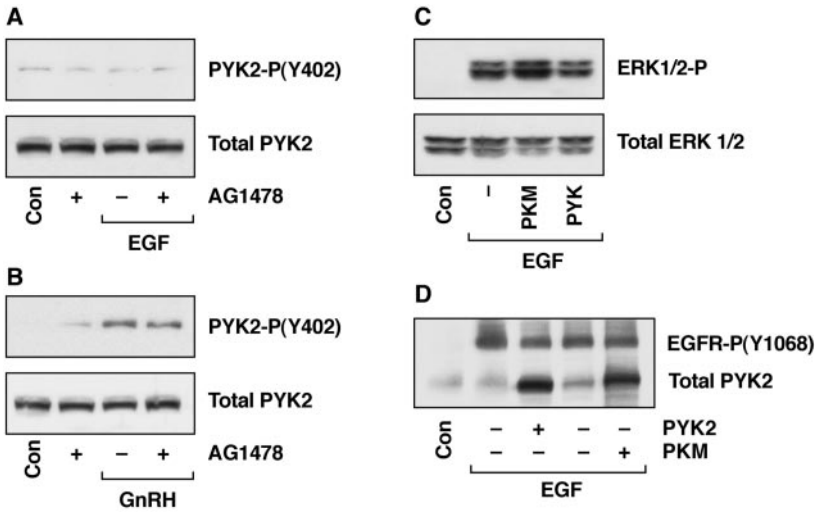

FIG. 11. **Pyk2 acts upstream of the EGF-R during GnRH stimulation.** *A*, effect of EGF (50 ng/ml for 5 min) on Pyk2 phosphorylation. *B*, lack of effect of AG1478 (100 nM) on GnRH-induced Pyk2 activation. Serum-starved GT1–7 cells were pretreated with inhibitors for 20 min before stimulation with agonists. Cells were collected in Laemmli sample buffer, and immunoblot analysis was done as described under "Experimental Procedures." *C* and *D*, lack of effects of dominant negative Pyk2 (PKM) and Pyk2 overexpression on EGF-induced phosphorylation of ERK1/2 and EGF-R at Tyr^{1068}.

operative during GPCR-induced MAPK signaling in neuronal cells.

The requirement for growth factor receptor transactivation in GnRH-induced ERK1/2 phosphorylation in other cell types is controversial. Whereas observations in immortalized pituitary gonadotrophs (αT3–1 cells) and COS-7 cells expressing GnRH receptors have suggested that GnRH-induced ERK1/2 activation involved transactivation of the EGF-R, more recent studies found no role of EGF-R transactivation in the phosphorylation of MAPK in αT3–1 cells (9) and HeLa cells expressing GnRH-R (23). Our data show that EGF-Rs are abundantly expressed in GT1–7 cells and, when stimulated, undergo marked autophosphorylation, leading to activation of ERK1/2. More significantly, transactivation of the EGF-R kinase is essential for GnRH-induced ERK1/2 activation in GT1–7 neurons, which is completely prevented by the selective EGF-R kinase inhibitor AG1478. Furthermore, GnRH causes selective phosphorylation of the EGF-R at Tyr^{1173}, a target site for Src action (35) and at Tyr^{1068}, a binding site for the Grb2/Src homology 2 domain (36) (Fig. 6).

Whereas involvement of the EGF-R in GPCR-mediated ERK1/2 activation is well documented (14, 17, 18, 22), the molecular mechanisms responsible for this cascade are not clearly defined. Depending on the cell type, EGF-R transactivation has been reported to be mediated by G_i protein $\beta\gamma$-subunits (40, 41), Ca^{2+} (26, 30), PKC (10, 37, 42), and heparin-binding EGF (HB-EGF) released by matrix metalloproteinases (43, 44). However, the latter does not appear to be a universal mechanism for transactivation of the EGF-R by GPCRs (17, 45). Recently, the metabotropic glutamate receptor-5 has been shown to directly interact with the EGF-R, bypassing phospholipase Cβ, PKC, and Ca^{2+} (46). In the present study, the use of pharmacological inhibitors and constitutively active and dominant negative mutants of relevant signaling molecules has defined PKC, Src, and Pyk2 as critical factors in GnRH-mediated EGF-R transactivation and the consequent increase in ERK1/2 phosphorylation.

Tyrosine kinases implicated in cell signaling include Src family kinases, RTKs such as the EGF-R, the FAK family, Pyk2, and JAK kinases. Pyk2 belongs to the FAK family and is activated by tyrosine phosphorylation in response to several GPCRs (39) as well as by stress stimuli (47) and membrane depolarization (36). Pyk2 has also been implicated in the regulation of ion channels (48), cell adhesion and motility, neurite outgrowth (38, 39), and the induction of long term potentiation in CA1 hippocampal cells (49). Under various experimental conditions, Pyk2 has been shown to participate

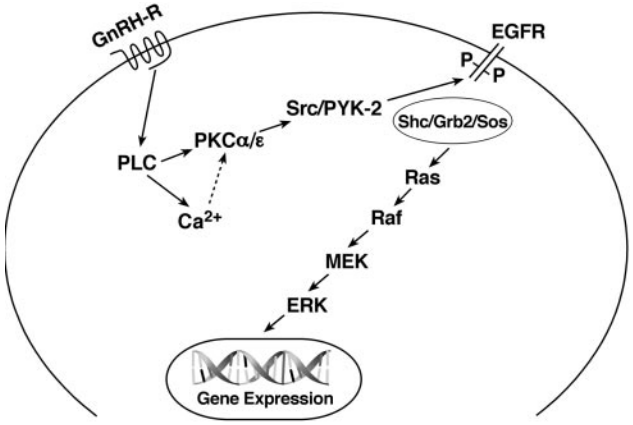

FIG. 12. **Schematic representation of the signaling pathways involved in GnRH action in GT1–7 hypothalamic neurons.** GnRH-R stimulation leads to activation of G_q/phospholipase C and translocation of PKCα/ϵ to the membrane. Agonist stimulation increases the association of PKC with Src and that of Src with Pyk2. The activated Src-Pyk2 complex associates with and activates EGF-R, leading to recruitment of adaptor proteins, such as Shc-Grb2-SOS, and subsequent activation of the ERK1/2 cascade.

in the activation of all three major MAPKs: ERK1/2 (26, 48, 50–52), p38 MAPK (28), and Jun N-terminal kinase (27). However, no evidence about the role of Pyk2 in GnRH-induced ERK1/2 activation has been available. Our data have established that GnRH causes marked stimulation of Pyk2 phosphorylation and that overexpression of a kinase-inactive Pyk2 mutant impairs ERK1/2 activation by GnRH and PMA (Figs. 9 and 10). We have also demonstrated that Pyk2 acts upstream of the EGF-R, since EGF failed to activate Pyk2 and dominant negative Pyk2 had no effect on EGF-induced responses. Moreover, GnRH-induced phosphorylation of Pyk2 was not affected by AG1478 (Fig. 11).

The present data also show that GnRH enhances the association of Pyk2 with c-Src (Fig. 10), an interaction that results from binding of the autophosphorylated Tyr^{402} of Pyk2 to the Src homology 2 domains of c-Src (50–52). Expression of wild type Pyk2 induces phosphorylation of Shc and increases its association with Grb2 (52), a finding consistent with GnRH-induced phosphorylation of the EGF-R at Tyr^{1068} (Fig. 6). On the other hand, a mutant form of Pyk2 that cannot complex with c-Src behaves as a dominant negative inhibitor of GPCR-stimulated ERK1/2 activation (50). Our studies using both the selective Src inhibitor, PP2, and the C-terminal Src kinase,

Csk, have demonstrated the essentiality of Src in the activation of ERK1/2 by GnRH and PMA (Fig. 8). In contrast, the lack of effect of Src inhibition on EGF-induced ERK1/2 activation indicates that Src acts upstream of EGF-R in GT1–7 cells. Since Src was essential for GnRH-induced phosphorylation of the EGF-R, these findings indicate that activation of Src/Pyk2 has a critical role in transducing signals from the GnRH-R to EGF-R transactivation in GnRH neuronal cells. These results are consistent with recent studies in fibroblasts from knockout mice showing that Src kinases are critical for activation of Pyk2 and that Src and Pyk2 are indispensable for EGF-R activation by GPCRs (31). In contrast, ERK1/2 activation by GnRH in αT3–1 pituitary gonadotrophs was independent of Pyk2 as well as EGF-R transactivation. Instead, it was primarily mediated through the direct activation of Raf-1 by PKC and to a lesser extent by Ras activation that was dependent on dynamin and Src (9). Similarly, whereas ERK1/2 activation by endothelin-1 in rat mesangial cells involved Pyk2, it was independent of EGF-R activation (28). It is clear that the matrix of signaling molecules utilized during GPCR stimulation is highly variable among different cell types, in which several specific patterns of interactions and phosphorylations are now becoming evident.

Earlier studies on the role of PKC isoforms in GnRH action have demonstrated activation of PKCδ and -ϵ (53) and PKCβ2, -δ, -ϵ, and -ζ in αT3–1 cells (54) and activation of PKCα and -β in rat pituitary cells (55). However, no information was available about the specific PKC isoform(s) involved in GnRH-induced ERK activation in GT1–7 cells. We found that GT1–7 cells contain PKC isoforms α, δ, ϵ, and λ and that both GnRH and PMA increased the translocation of PKCα and -ϵ to the cell membranes and enhanced their association with Src (Figs. 3 and 8). Moreover, GnRH-stimulated phosphorylation of Pyk2 and ERK1/2 in GT1–7 cells is primarily dependent on PKC, since both pharmacological PKC inhibition and PKC depletion by PMA abolished ERK1/2 activation by GnRH and PMA but not by EGF (Figs. 2, 7, and 8). Overexpression of dominant negative PKCα also attenuated ERK1/2 activation by GnRH (Fig. 4), but not by EGF (data not shown). These data suggest that whereas PKC is an important mediator of GnRH-induced signals, its stimulatory action is upstream of the EGF-R in GT1–7 cells. GPCR-mediated activation of Pyk2 and ERK1/2 is reported to be dependent on both Ca^{2+} (26, 30, 41, 50) and PKC (19, 37, 39, 51). PKC is also known to cause activation of Src (24) and the EGF-R (10, 24, 44, 45) in several cell types. In fact, PKCα and -δ undergo direct physical and functional interactions with the EGF-R and Pyk2, respectively (56, 57). Thus, PKC can stimulate signaling cascades by targeting a variety of intermediary proteins.

In many cells, the pathways of GPCR- and RTK-mediated ERK1/2 activation converge primarily at the level of the EGF-R (20, 22, 41, 58). Following agonist-induced tyrosine phosphorylation of the EGF-R, the signaling pathways involved in ERK1/2 activation by GPCRs and EGF-Rs appear to be identical (14, 20). This also applies to the action of GnRH in GT1–7 cells. In conclusion, our results show that GnRH causes rapid phosphorylation of ERK1/2 through transactivation of the EGF-R. Such cross-regulation between the GnRH and EGF receptors occurs through the rapid activation of PKC, Src, and Pyk2 by GnRH. Our data support the view that agonist stimulation of neuronal GnRH receptors induces the assembly of a multiprotein signaling complex that includes PKCα/ϵ, Src/Pyk2, and the EGF-R. A summary of the manner in which GnRH causes phosphorylation of ERK1/2 in GT1–7 cells is shown in Fig. 12.

REFERENCES

1. Stojilkovic, S. S., and Catt, K. J. (1995) *J. Neuroendocrinol.* **7,** 739–757
2. Mores, N., Krsmanovic, L. Z., and Catt, K. J. (1996) *Endocrinology* **137,** 5731–5734
3. Krsmanovic, L. Z., Stojilkovic, S. S., Mertz, L. M., Tomic, M., and Catt, K. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 3908–3912
4. Martinez de la Escalera, G., Choi, A. L., and Weiner, R. I. (1992) *Endocrinology* **131,** 1397–1402
5. Krsmanovic, L. Z., Mores, N., Navarro, C. E., Saeed, S. A., Arora, K. K., and Catt, K. J. (1998) *Endocrinology* **139,** 4037–4043
6. Hery, M., Francois-Bellan, A. M., Hery, F., Deprez, P., and Becquet, D. (1997) *Endocrine* **7,** 261–265
7. Marinissen, M. J., and Gutkind, J. S. (2001) *Trends Pharmacol. Sci.* **22,** 368–376
8. Naor, Z., Benard, O., and Seger, R. (2000) *Trends Endocrinol. Metab.* **11,** 91–99
9. Benard, O., Naor, Z., and Seger, R. (2001) *J. Biol. Chem.* **276,** 4554–4563
10. Grosse, R., Roelle, S., Herrlich, A., Hohn, J., and Gudermann, T. (2000) *J. Biol. Chem.* **275,** 12251–12260
11. Han, X-B., and Conn, P. M. (1999) *Endocrinology* **140,** 2241–2251
12. Mulvaney, J. M., Zhang, T., Fewtrell, C., and Roberson, M. S. (1999) *J. Biol. Chem.* **274,** 29796–29804
13. Reiss, N., Levi, L. N., Shachm, S., Harris, D., Seger, R., and Naor, Z. (1997) *Endocrinology* **138,** 1673–1682
14. Luttrell, L. M., Daaka, Y., and Lefkowitz, R. J. (1999) *Curr. Opin. Cell Biol.* **11,** 177–183
15. Mondorf, U. F., Geiger, H., Herrero, M., Zeuzem, S., and Piiper, A. (2000) *FEBS Lett.* **472,** 129–132
16. Roudabush, F. L., Pierce, K. L., Maudsley, S., Khan, K. D., and Luttrell, L. M. (2000) *J. Biol. Chem.* **275,** 22583–222589
17. Saito, Y., and Berk, B. C. (2001) *J. Mol. Cell Cardiol.* **33,** 3–7
18. Maudsley, S., Pierce, K. L., Zamah, A. M., Miller, W. E., Ahn, S., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M. (2000) *J. Biol. Chem.* **275,** 9572–9580
19. Soltoff, S. P. (1998) *J. Biol. Chem.* **273,** 23110–23117
20. Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich. A. (1997) *EMBO J.* **16,** 7032–7044
21. Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M. (1999) *J. Biol. Chem.* **274,** 13978–13984
22. Gschwind, A., Zwick, E., Prenzel, N., Leserer, M., and Ullrich, A. (2001) *Oncogene* **20,** 1594–1600
23. Hislop, J. N., Everest, H. M., Flynn, A., Harding, T., Uney, J. B., Troskie, B. E., Millar, R. P., and McArdle, C. A. (2001) *J. Biol. Chem.* **276,** 39685–39694
24. Levi, N., Hanoch, T., Benard, O., Rozenblat, M., Harris, D., Reiss, N., Naor, Z., and Seger, R. (1998) *Mol. Endocrinol.* **12,** 815–824
25. Liu, F., Austin, D. A., Mellon, P. L., Olefsky, J. M., and Webster, N. J. G. (2002) *Mol. Endocrinol.* **16,** 419–434
26. Eguchi, S., Iwasaki, H., Inagami, T., Numaguchi, K., Yamakawa, T., Motley, E. D., Owasa, K. M., Marumo, F., and Hirata, Y. (1999) *Hypertension* **33,** 201–206
27. Matsubara, H., Shibasaki, Y., Okigaki, M., Mori, Y., Masaki, H., Kosaki, A., and Tsutsumi, Y. (2001) *Biochem. Biophys. Res. Commun.* **282,** 1085–10891
28. Sorokin, A., Kozlowski, P., Graves, L., and Philip, A. (2001) *J. Biol. Chem.* **276,** 21521–21528
29. Iwasaki, H., Shichiri, M., Marumo, F., and Hirata, Y. (2001) *Endocrinology* **142,** 564–572
30. Keely, S. J., Calandrella, S. O., and Barrett, K. E. (2000) *J. Biol. Chem.* **275,** 12619–12625
31. Andreev, J., Galisteo, M. L., Kranenburg, O., Logan, S. K., Chiu, E. S., Okigaki, M., Cary, L. A., Moolenaar, W. M., and Schlessinger, J. (2001) *J. Biol. Chem.* **276,** 20130–20135
32. Soh, J-W., Lee, E. H., Prywes, R., and Weinstein, I. B. (1999) *Mol. Cell. Biol.* **19,** 1313–1324
33. Kaiser, U. B., Conn, P. M., and Chin, W. W. (1997) *Endocr. Rev.* **18,** 46–70
34. Krsmanovic, L. Z., Mores, N., Navarro, C. E., Tomic, M., and Catt, K. J. (2001) *Mol. Endocrinol.* **15,** 429–440
35. Wright, J. D., Reuter, C. W., and Weber, M. J. (1996) *Biochim. Biophys. Acta* **1312,** 85–93
36. Zwick, E., Wallasch, C., Daub, H., and Ullrich, A. (1999) *J. Biol. Chem.* **274,** 20989–20996
37. Shah, B. H., and Catt, K. J. (2002) *Mol. Pharmacol.* **61,** 343–351
38. Ivankovic-Dikic, I., Gronroos, E., Blaukat, A., Barth, B. U., and Dikic, I. (2000) *Nat. Cell Biol.* **2,** 574–581
39. Avraham, H., Park, S-Y., Schinkmann, K., and Avraham, S. (2000) *Cell. Signal.* **12,** 123–133
40. Herrlich, A., Daub, H., Knebel, A., Herrlich P., Ullrich A., Schultz, G., and Gudermann, T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8985–8990
41. Luttrell, L. M., Della Rocca, G. J., Biesen, T. V., Luttrell, D. K., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 4637–4644
42. Tsai, W., Morielli, A. D., and Peralta, E. G. (1997) *EMBO J.* **16,** 4597–4605
43. Pierce, K. L., Tohgo, A., Ahn, S., Field, M. E., Luttrell, L. M., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276,** 23155–23160
44. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402,** 884–888
45. Grewal, J. S., Luttrell, L. M., and Raymond, J. R. (2001) *J. Biol. Chem.* **276,** 27335–27344
46. Peavy, R. D., Chang, M. S., Sanders-Bush, E., and Conn, P. J. (2001) *J. Neurosci.* **21,** 9619–9628
47. Tokiwa, G., Dikic, I., Lev, S., and Schlessinger, J. (1996) *Science* **273,** 792–794
48. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) *Nature* **376,** 737–745
49. Huang, Y., Lu, W., Ali, D. W., Pelkey, K. A., Pitcher, G. M., Lu, Y. M., Aoto, H., Roder, J. C., Sasaki, T., Salter, M. W., and Mcdonald, J. F. (2001) *Neuron* **29,** 485–496

50. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) *Nature* **383,** 547–550
51. Li, X., Dy, R. C., Cance, W. G., Graves, L. M., and Earp, H. S. (1999) *J. Biol. Chem.* **274,** 8917–8924
52. Blaukat, A., Ivankovic-Dikic, I., Gronroos, E., Dolfi, F., Tokiwa, G., Vuori, K., and Dikic, I. (1999) *J. Biol. Chem.* **274,** 14893–14901
53. Harris, D., Reiss, N., and Naor, Z. (1997) *J. Biol. Chem.* **272,** 13534–13540
54. Poulin, B., Rich, N., Mas, J. L., Kordon, C., Enjalbert, A., and Drouva, S. V. (1998) *Mol. Cell. Endocrinol.* **142,** 99–117
55. Naor, Z., Dan-Cohen, H., Hermon, J., and Limor, R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4501–4504
56. Seedorf K., Shearman, M., and Ullrich, A. (1995) *J. Biol. Chem.*, **270,** 18953–18960
57. Wrenn, R. W. (2001) *Biochem. Biophys. Res. Commun.* **282,** 882–886
58. Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A. (1996) *Nature* **379,** 557–560